PROJECT ANNUAL REPORT
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Project Title: Molecular characterization and detection of *Fusarium oxysporum* f. sp. *lactucae* in lettuce seed and field soil.
Project Principal Investigator: Barry M. Pryor, University of Arizona
Cooperating personnel: Gladys C. Mbofung, University of Arizona

INTRODUCTION

Fusarium wilt caused by the fungus *Fusarium oxysporum* is one of the most destructive soil borne diseases of agricultural and horticultural crops worldwide. The fungus is commonly found in most soils and has a wide host range. Individual isolates of the fungus usually demonstrate a high degree of host specificity. In 1993, *Fusarium* wilt was reported on lettuce plants in the Fresno area in California, but it was only in 2001 that the disease was found in Arizona in five neighboring fields. Over the last two years, the disease has spread to 11 additional fields, clearly demonstrating its ability to spread to neighboring lettuce fields. The spread of this pathogen in lettuce fields throughout Arizona could have serious consequences as there are few options available to growers to combat the disease. Management methods currently in use include avoidance of infested fields, late planting and/or use of lettuce cultivars less susceptible to the disease. A substantial amount of research is immediately necessary to develop methods of detection that could be integrated into effective disease management programs.

The objectives of this study were:

1. To develop molecular-based methods for detecting and differentiating *F. oxysporum* f. sp. *lactucae* from other *Fusarium* species.
2. To develop molecular-based protocols for detecting this pathogen in lettuce seed and field soil.
3. To determine the genetic structure within the *lactucae* population in order to document how the fungus is introduced into Arizona and how the fungus is spread once introduced.

MATERIALS AND METHODS

Fusarium strains used in this study were obtained from three main sources: diseased lettuce roots, soil from infested and non-infested fields and some goodwill donors (table 1). Pathogenicity tests were performed on a select sample to determine their virulence on three lettuce cultivars.

For the molecular studies, the isolates were grown on potato dextrose agar and then transferred to *Alternaria* broth for further growth. Mycelia were harvested from the broth, lyophilized, and used to isolate DNA for polymerase chain reactions (PCR). Four genes and one intergenic region (histone H4, mitochondrial small subunit mtSSU, the translation elongation 1α factor EF-1α and the intergenic spacer region IGS, respectively) were chosen for sequencing. These genomic regions were selected based on the fact that they evolve faster and that at least two copies of each exist in the genome. In addition, PCR primers specific for these gene/regions were already available from previous studies.

The sequences obtained were used to resolve phylogenetic differences between the *lactucae* isolates and other formae speciales in the *Fusarium oxysporum* species complex.

In the detection study, single nucleotide polymorphisms in the IGS sequence were detected that differentiated pathogenic from non-pathogenic *Fusarium* strains. These sites were chosen and the restriction enzymes specific for these sites determined. A restriction enzyme site for *Hind* III was chosen and adapters were synthesized for this site. A primer was designed specific for the adapter and, together with one of the previously available primers, were used to selectively amplify a 1270 base pair fragment of the *Hind* III restricted IGS region only from the pathogenic isolates (*lactucae* isolates).

To determine the sensitivity of this method, 10-fold serial dilutions of *F. o. lactucae* total genomic DNA were prepared in TE buffer (concentrations ranged from 10 ng/µl to 1fg/µl), and the capacity of the primer pair to direct the amplification of the target DNA fragment from the decreasing concentrations of DNA was determined. The specificity of the primer pair was tested with total genomic DNA (10ng/µl) from six fungal species. Detection of *Fusarium oxysporum* f. sp. *lactucae* in soil, lettuce tissue and seed was tested using DNA extracts spiked with decreasing amounts of total genomic DNA with the addition of skim milk to overcome PCR inhibitors released during extraction.

Studies to determine the population structure of the strains of *Fusarium oxysporum* causing disease on lettuce were carried out using five microsatellite primers. The sample population consisted of *lactucae* strains from regions around the world where lettuce wilt has been reported.

RESULTS

Phylogenetic analysis of the H4 and mitochondrial small subunit (mtSSU) gene sequences resulted in low resolution among the isolates tested. Analysis of the EF-1α sequences gave a better resolution however, grouped some non-lettuce-specific isolates with the *lactucae* isolates. The IGS sequences obtained were sufficiently variable to allow for discrimination between the *lactucae* isolates from the other formae speciales. Phylogenetic reconstructions using these sequences revealed that the *lactucae* isolates constituted a closely related monophyletic group distinguishable from the other formae speciales and the non-pathogenic isolates obtained from soil (Fig. 1). Close examination of the sequences revealed single nucleotide polymorphisms as well as short insertions and deletions existing between the pathogenic and non-pathogenic strains. In addition, the IGS phylogeny revealed two distinct lineages for the *lactucae* isolates: one composed of isolates belonging to Race 1 (from Arizona and California) and a second lineage composed of Race 2 isolates (from Japan). These results indicate that races of F. o. f.sp. *lactucae* have evolved independently of each other and are unrelated. To date, three races have been reported; only Race 1 is found in the USA but Races 1, 2, and 3 are present in Japan.

Based on the single nucleotide polymorphisms, a *Hind III* restriction site was selected and used to develop adapters and oligomers for the amplification of a *lactucae* specific fragment based on the polymerase chain reaction. The restriction enzyme cut only DNA from the pathogenic isolates resulting in a 1270 bp fragment (Fig. 2). The adapter and the CNL12 primer pair re-amplified this fragment resulting in an amplification product only from the pathogenic *Fusarium* isolates and not from any other

Fusarium isolate or species (Fig. 3). The lowest concentration of total genomic DNA from which the target fragment was amplified was 20 fg (data not shown).

DNA extracts were prepared from un-contaminated seed, soil and lettuce leaf tissue. When total genomic DNA from F.o. f.sp. *lactucae* was added to these extracts at a concentration of 10 ng/µl, the target DNA sequence was not initially amplified, which suggests that there are inhibitors to PCR present in these extracts (Fig. 4). However, when 0.2% skim milk were added to the extracts, PCR of the IGS region preceded properly, which reveals that the addition of skim milk could successfully be used to overcome PCR inhibitors released from tissues during DNA isolation (Fig. 4). Thus, these results show that the method developed could be used for rapid, specific, and sensitive detection of the fungus in samples of seed, soil, and lettuce tissue. However, this method is designed to work for race 1 isolates because race 2 isolates do not possess the *Hind* III restriction in the IGS sequence. A different restriction enzyme will be used for the other two races of the pathogen.

In preliminary studies using two microsatellite primers, cluster analysis of the fingerprint data primers revealed genetic variability within the *lactucae* isolates from California, but no variation among the Arizona isolates (Fig 5). These results suggest that the Arizona isolates resulted from a single introduction from California. These studies have been expanded to include five microsatellite primers, several more isolates from newly infested fields in Arizona, and several more isolates from California, Italy, and Taiwan. These additional fingerprint analyses are ongoing.

FUTURE DIRECTIONS

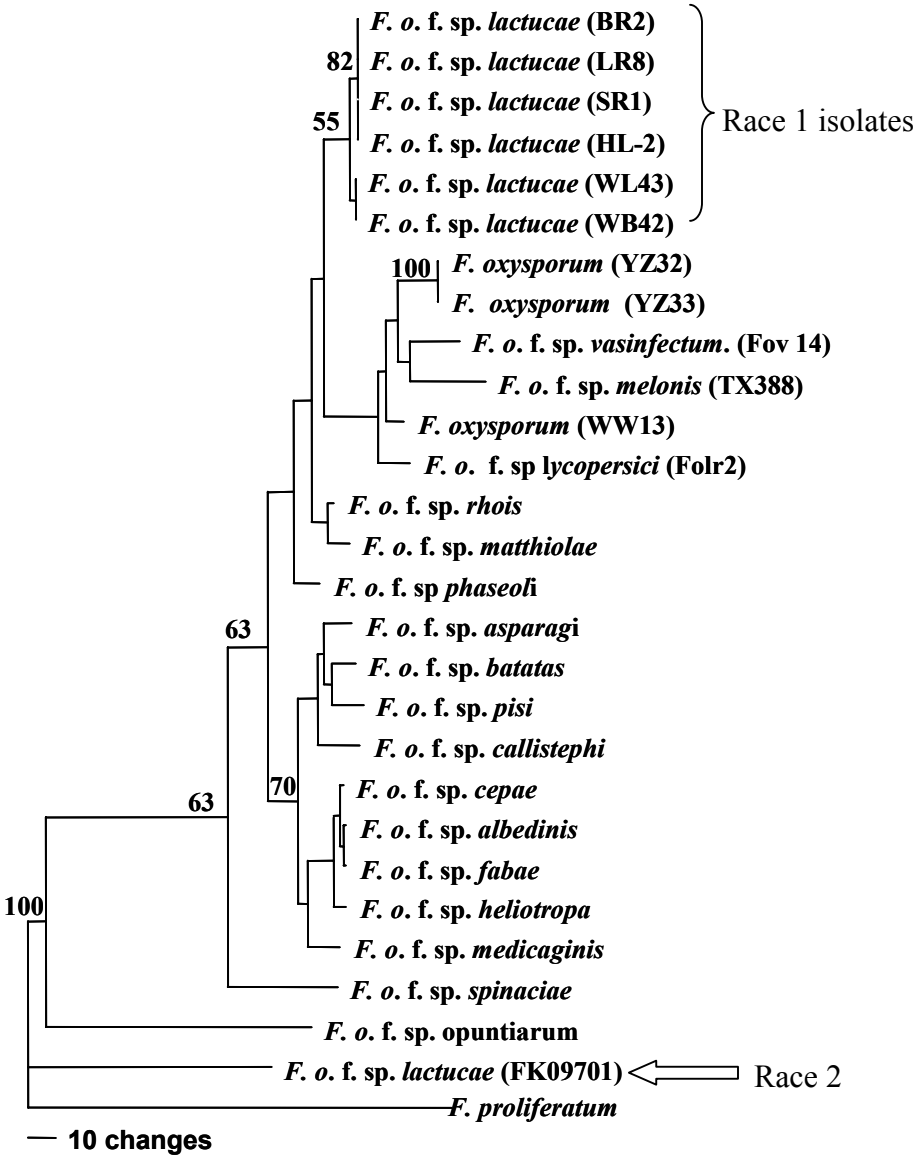
Results from this year have revealed a unique genetic region that contains DNA variation specific only to *Fusarium* isolates pathogenic to lettuce. Based upon these results, a PCR method has been developed that detects only the pathogenic isolates and not any other *Fusarium* species. This method is suitable for detecting the pathogen in samples of seed, soil, and lettuce tissue. The focus of the proposed 2003-2004 research will be to validate these finding in actual contaminated samples of seed, soil, and tissue, and to determine the sensitivity of detection in these environmental samples. Once this method has been validated, a survey of lettuce seed obtained from growers in the Yuma area will be undertaken to determine the presence and level of infestation of lettuce seed currently used. In addition, seed obtained from other areas of lettuce seed production, such as the Huron area of California and possibly from Japan will be analyzed for possible infestation. In addition, a more detailed analysis of pathogenic *Fusarium* isolates obtained from recently-infested fields in Arizona will be conducted to determine if these additional infestations represent new introductions of the pathogen or simply a spread of the pathogen that is already established in select fields.

Table 1. Isolates of *Fusarium oxysporum* forma specialis *lactucae* and other *formae speciales* used in this study. Also included are non-pathogenic soil isolates of *F. oxysporum* as well as *F. proliferatum* used as an out-group.

Isolate	Taxon and race	Host/substrate	Pathotype	origin
BR2	*F. o*. f. sp. *lactucae*	*Lactuca*	Pathogenic	Wellton, AZ
LR8	*F. o*. f. sp. *lactucae*	*Lactuca*	Pathogenic	Wellton, AZ
SR1	*F. o*. f. sp. *lactucae*	*Lactuca*	Pathogenic	Wellton, AZ
HL-2	*F. o*. f. sp. *lactucae*	*Lactuca*	Pathogenic	Fresno, CA
FK09701	*F. o*. f. sp. *lactucae*	*Lactuca*	Pathogenic	Japan
WL43	*F. o*. f. sp. *lactucae*	Soil	Pathogenic	Wellton, AZ
WB42	*F. o*. f. sp. *lactucae*	Soil	Pathogenic	Wellton, AZ
YZ32	*F. oxysporum*	Soil	nonpathogenic	Yuma, AZ
YZ33	*F. oxysporum*	Soil	nonpathogenic	Yuma, AZ
WW13	*F. oxysporum*	Soil	nonpathogenic	Wellton, AZ
Fov14	*F. o*. f. sp. *vasinfectum*	*Gossypium*	nd	King County, CA
TX388	*F. o*. f. sp. *melonis*	*Cucumis*	nd	Texas
Fol r2	*F. o*. f. sp. *lycopersici*	*Lycopersicon*	nd	California
FoA50	*F. o*. f. sp. *asparagi*	*Asparagus*	nd	Australia
NRRL22536	*F. o*. f. sp. *callisteph*i	*Callistephus*	nd	NRRL[a]
NRRL22539	*F. o*. f. sp. *chrysanthemi*	*Chrysanthemum*	nd	NRRL[a]
NRRL22535	*F. o*. f. sp. *batatas*	*Ipomoea*	nd	NRRL[a]
NRRL26871	*F. o*. f. sp. *spinaciae*	*Spinacia*	nd	NRRL[a]
NRRL26445	*F. o*. f. sp. *phaseoli*	*Phaseolus*	nd	NRRL[a]
NRRL28934	*F. o*. f. sp. *opuntiarum*	Opuntia	nd	NRRL[a]
NRRL25231	*F. o*. f. sp. *pisi*	*Pisum*	nd	NRRL[a]
NRRL22546	*F. o*. f. sp. *medicaginis*	Medicago	nd	NRRL[a]
NRRL22538	*F. o*. f. sp. *cepae*	*Allium*	nd	NRRL[a]
NRRL22431	*F. o*. f. sp. *vasinfectum*	*Gossypium*	nd	NRRL[a]
NRRL26622	*F. o*. f. sp. *albedinis*	*Phoenix*	nd	NRRL[a]
NRRL26412	*F. o*. f. sp. *heliotropa*	*Heliotropium*	nd	NRRL[a]
NRRL26411	*F. o*. f. sp. *fabae*	*Vicia*	nd	NRRL[a]
NRRL26227	*F. o*. f. sp. *rhois*	*Rhus*	nd	NRRL[a]
NRRL22545	*F. o*. f. sp. *matthiolae*	*Matthiola*	nd	NRRL[a]
31.X4	*F. proliferatum*	*Curcubita*	nd	Yuma, AZ

[a]NRRL= National Center for Agricultural Utilization Research, Preoria, IL.
[b] Strains nonpathogenic to lettuce
nd= not determined

Figure 1. phylogram generated with IGS sequence data. Bootstrap values are based on 100 replications. The tree is rooted with *Fusarium proliferatum*. The tree length is 696, consistrency index is 0.816 and the retention index is 0.713.



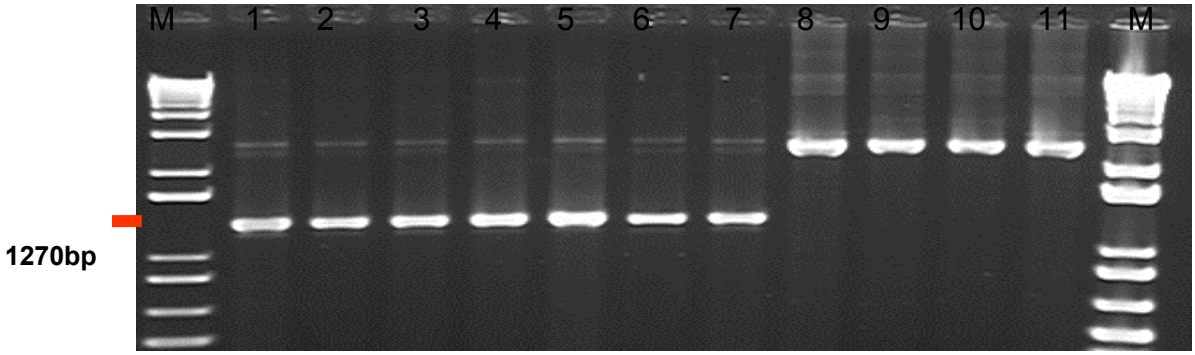


Fig. 2. Restriction digest of the intergenic spacer region (IGS) product obtained from Polymerase chain reaction. Lanes 1 to 7, pathogenic isolates (BR2, HL-2, YB41, HL-1, WL32, Y06, YB13), lanes 8 to 11, non-pathogenic isolates, (YZ42, WW13, YZ31, YZ334).

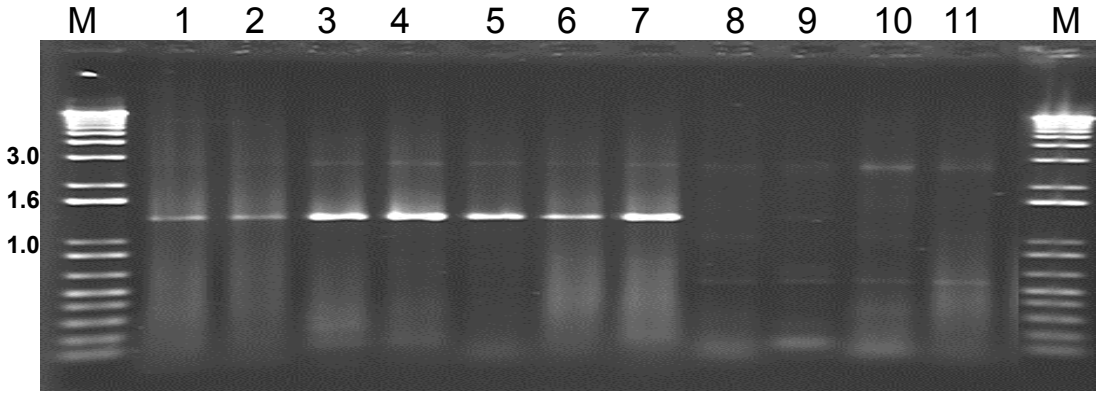


Fig. 3. Amplified fragment generated from the restriction/ligation using the adapter primer (H-0) and the universal primer, CNL12. Lanes 1 to 7, isolates pathogenic on lettuce (BR2, HL-2, YB41, HL-1, WL32, Y06, YB13); lanes 8 to 11, nonpathogenic isolates (YZ42, WW13, YZ31, YZ334).

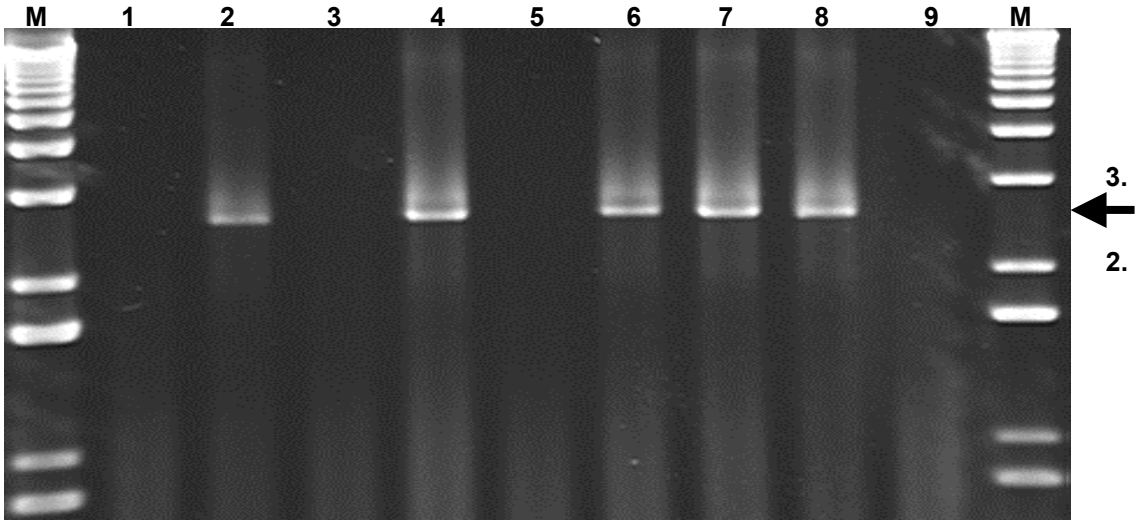


Fig. 4. Amplification of the IGS region from extracts of soil sample, lettuce leaf tissue and seed with the addition of skimmed milk. Lanes 1 and 2, soil samples; Lanes 3 and 4, represent leaf tissue; lanes 5 and 6, lettuce seeds; 20 µl of genomic DNA at 10 ng/ µl were added to samples in lanes 2, 4, and 6. Lanes 7 and 8 contain pathogenic isolates LR2 and HL-1 respectively. Lane 9 contains no DNA. Lanes **M** contain the 1-Kb plus DNA ladder.

Fig. 5. Phylogram obtained with the microsatellite fingerprint data showing the relationship between the California and the Arizona isolates. Clade A contains one California isolate (HL-2) and Arizona *lactucae* isolates. Clade B is made up of California isolates and C is made up of strains of uncertain affinity from California and Arizona.